EXPRESSION OF HOMEOBOX-CONTAINING GENES IN FRESHWATER SPONGES

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Homeoboxes have been particularly valuable to identify genes involved in development. This prompted us to look for homeobox-containing genes in sponges, the most primitive metazoans, and to explore the potential role of these genes in sponge development. Using RT-PCR, we have shown that two homeobox-containing genes, *EmH-3* and *prox1* are present in five freshwater sponge species: *Ephydatia muelleri, E. fluviatilis, Spongilla lacustris, Eunapius fragilis* and *Trochospongilla horrida. EmH-3* is expressed differentially during gemmule germination and hatching in *E. muelleri* as well as in *E. fluviatilis*. The expression pattern of *EmH-3* suggests a role during cell differentiation. Hydroxyurea, which specifically blocks the differentiation of choanocytes and the aquiferous system, seems not to affect the expression pattern of *EmH-3*. Contrary to *EmH-3*, prox1 is expressed almost at the same level throughout development. ❐ *Porifera,homeobox-containing genes, development, expression.*

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Homeobox-containing genes are important developmental genes that play a central role in the early development of a variety of organisms. It was thought for a time that they were only involved in spatial and temporal organisation in segmented animals, whereas it is now known that they are also active in non-segmental organisms and systems, and are implicated in axial patterning and cell-fate decisions during differentiation (Davidson, 1995; Dolecki et al., 1986; Garcia et al., 1993; Lawrence & Morata, 1994; Salser & Kenyon, 1996).

Homeobox-containing genes have been identified throughout the animal kingdom, from primitive phyla such as cnidarians, nematodes, flatworms and more recently sponges, to chordates (Ruddle et al., 1994). They have been isolated both from freshwater sponges (Coutinho et al., 1994; Richelle et al., 1998; Seimiya et al., 1994; Seimiya et al., 1997), and from marine sponges (Degnan et al., 1995; Kruse et al., 1994). The presence of homeobox-containing genes in Porifera is of particular interest, and of evolutionary significance, as sponges are considered to be the most primitive metazoans: they do not display any type of symmetry nor polarity, nor do they contain distinct organs or a nervous system. Therefore, elucidating the structure, function and role of homeobox-containing genes in sponges is essential to comprehend the evolution of these genes in metazoans.

As previously reported, we have isolated and sequenced three homeobox-containing genes: *EfH-1* and *EfH-2* from *Ephydatia fluviatilis* using the PCR reaction and degenerated *Antennapedia* primers (Coutinho et al., 1994), and *EmH-3* from *Ephydatia muelleri* by screening an *E. muelleri* genomic library with *EfH-1* (Richelle et al., 1998).

The nucleotide and predicted amino acid sequences of *EfH-1* and *EfH-2* are very different whereas *EfH-1* is very similar to *EmH-3* (85%-86%).

The comparison of *EfH-1* and *EmH-3* homeodomains with all known sponge homeodomains *prox1*, *prox2*, *prox3* from *E. fluviatilis* (Seimiya et al., 1994), *SHOX* from *Geodia cydonium* (Kruse et al., 1994), *SpoxTA1* from *Tethya aurantia*, and *SpoxH1* and *SpoxH2* from *Haliclona* sp. (Degnan et al., 1995) has revealed the highest similarity with *prox2* and *SpoxTA1* (Table1). *EfH-1* and *EmH-3* share a lesser degree of similarity with *prox3* and *prox1*, and are not more closely related to them than to *Cnox3*, *Cnox2* and *Cnox1* from *Hydra* (Schummer et al., 1992; Shenk et al., 1993). They exhibit only a low level of similarity (19%) with *SHOX* homeodomain which seems at present not to belong to homeobox genes as it does not contain the critical sequence of standard homeodomains (Seimiya et al., 1998).

TABLE 1. Levels of similarity between sponge and hydra homeodomains in percent of identical amino acids including conservative substitutions.

Gene	*EfH-1* (37 aa)	*EmH-3*
prox2	92	98
SpoxTA1 (23aa)	96	96
prox3	73	70
SpoxH2 (23aa)	74	70
prox1	67	70
SpoxH1 (23aa)	52	52
SHOX	19	17
Cnox3	70	68
Cnox2	70	66
Cnox1	65	60

Phylogenetic studies have shown that *EmH-3* is closer to metazoan homeodomains than to those of yeast/ fungi and plants (Richelle et al., 1998). *EfH-1*, *EmH3*, *prox2* and probably *SpoxTA1* are representatives of the *Hox11/ Om* (1D) class; *prox1*, *prox3* and *SpoxH2* representatives of the *NK-3*, *msh* and *Chox7* class respectively and *SpoxH1* may be a representative of the *Antp*-class (Degnan et al., 1995). Nevertheless, no clustered homeobox genes have yet been reported in sponges.

A realignment of msh related genes by Master et al. (1996) has indicated that all four *NK*-class homeoboxes from *D. melanogaster* clustered with the sponge homeoboxes *prox1*, *prox2* and *prox3* to the exclusion of all other homeodomain family. The *NK* family is a large widespread family of non-clustered genes that appears to have been conserved throughout the evolution of animals and may be involved in specifying cell fate rather than specifying regional patterns (Shenk & Steele, 1993).

In the present study, we investigate the occurrence of *EmH-3* and *prox1* genes in three freshwater sponge species, common in Belgium: *Spongilla lacustris*, *Eunapius fragilis* and *Trochospongilla horrida* in addition to *E. fluviatilis* and *E muelleri* from which they where initially isolated. The expression of these genes was followed during gemmule germination and hatching. The effect of hydroxyurea on the expression of *EmH-3* was analyzed.

MATERIALS AND METHODS

SPONGE CULTURE. sponges were raised in the laboratory from gemmules in Petri dishes filled with sterile mineral medium (Rasmont, 1961) and incubated at 20°C. For some experiments, they were grown in mineral medium containing hydroxyurea at a final concentration of 100µg/ml (HU-medium). Finally some sponges were cultivated in the field. For this purpose, six-day-old sponges, hatched from gemmules on glass plates, were transferred to the outflow of a pond and were allowed to grow for several weeks.

RT-PCR EXPERIMENTS. This sensitive method for the detection and estimation of the levels of RNA transcripts was applied to analyse *EmH-3* gene expression during development. The expression of two other genes was followed in the same conditions: *prox1* homeobox-containing gene isolated from *E. fluviatilis*, known to be expressed at all stages of development for comparison (Seimiya et al., 1994); *EmA 1* actin gene isolated from *E. muelleri* as a control (Ducy, 1993). Total RNA was extracted at different stages of development, from gemmules to functional sponges, using TRIzol reagent as described in the instructions for use (Life Technologies). Before hatching, gemmules were collected and ground in a Potter homogeniser, on ice, in the presence of TRIzol reagent. After hatching, sponges were scraped and mechanically dissociated by pipetting. The gemmules were discarded, the dissociated cells were pelleted by low speed centrifugation (500g, 10mins, 4°C) and resuspended in TRIzol reagent. For sponges grown in the field, a small piece of the sponge was squeezed in cold mineral medium and the cells dissociated as described for laboratory sponge cultures. The quantity and purity of RNA was estimated by optical absorbance at 260nm and 280nm according to standard procedures (Sambrook et al., 1989). Its quality was checked on an agarose gel.

RT-PCR reactions were carried out using the Promega single tube, two-enzyme Access RT-PCR System which provides quick and reproducible analysis of even rare RNAs. All components necessary for RT-PCR were mixed in one tube with 10ng of total RNA and reverse transcription was automaticaly followed by PCR cycling without additional steps according to the manufacturer's protocol. The conditions were: 1 cycle of 45mins at 48°C; 1 cycle of 2mins at 94°C; 40 amplification cycles: 30sec at 94°C, 1min at 55°C (EmH-3, EmA 1) or 57°C (prox1) and 2mins at 68°C; followed by a final extension cycle of 7mins at 68°C. The amplification products were analysed by agarose gel (1%) electrophoresis of 10% of the total reaction.

The primers were supplied by Eurogenetec (Belgium). They were all gene specific and designed to flanked introns in order to discriminate between products that had been amplified from RNA and those that had been amplified from DNA. 1) For the study of *EmH-3*, the upstream primer, 5'-ATGGACAACTGCAGGG GTGA-3', was complementary to nt 1-20 of the first exon of the genomic sequence and the downstream primer, 5'-CATTCTCCTATTTTG GAACC-3', was complementary to nt 716-736 of the third exon containing the homeobox. 2) For the study of *prox1*, primers were those chosen by the authors Seimiya et al. (1994): the upstream primer, 5'-GGACAGATACGCTTCCGATCT-3', was complementary to nt 19-39 of the genomic sequence of the first exon and the downstream primer, 5'-ATATCGTCTGTTCTGAAA CCA -3', was complementary to nt 347-367 of the second exon. 3) For the study of *EmA 1*: the upstream primer, 5'-AACTGGGACGACATGG AGAA-3', was complementary to nt 15-35 of the published *EmA 1* Actin cDNA sequence (Ducy, 1993) and the downstream primer, 5'-GATCCA GACACTGTACTTGC-3', was complementary to nt 787-807. According to the author, there must be at least one intron between the sequences chosen for the two primers.

The nature of the amplified products was checked by digestion with specific restriction enzymes. The length of transcripts were: about 440bp for *EmH-3,* 240bp for *prox1*, 390bp for *prox2* and 792bp for *EmA 1.*

RESULTS

Gemmules hatched after 3-4 days incubation according to the species. Subsequently, choanocytes and aquiferous system became differentiated and the osculum appeared around 4-5 days of incubation. Seven-day-old sponges were considered to be fully functional.

In HU-medium, hatching was postponed by about 2 days and sponges had a typical hollow-dome structure (Rozenfeld & Rasmont, 1976). Neither choanocytes nor an aquiferous system were differentiated.

The investigation of *EmH-3* and *prox1* in *S. lacustris*, *E. fragilis*, *T. horrida* indicated that these genes were expressed in fully functional sponges in the three species as in *E. muelleri* and *E. fluviatilis* (Fig. 1). However, as far as *EmH-3* expression was concerned, there was a noticeable difference between the length of *E. fluviatilis* transcripts and those of *S. lacustris*, *E. fragilis* and *T. horrida* (Fig. 1A). The latter were

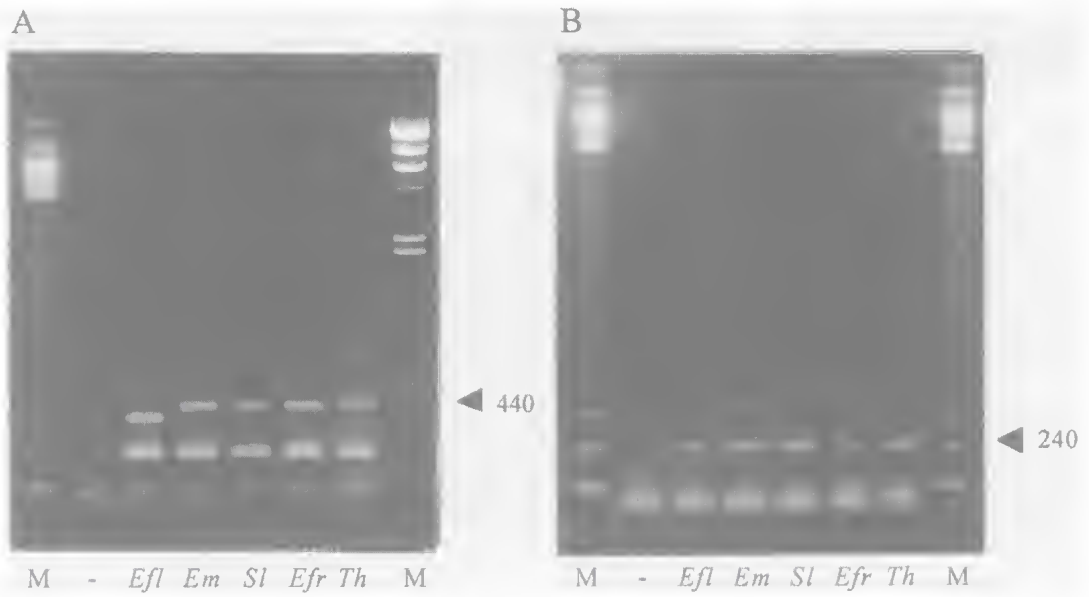

FIG.1. Expression of homeobox-containing genes in five freshwater species. Amplified products of RT-PCR of total RNA isolated from 7-day-old sponges. A, expression of *EmH-3* gene. B, expression of *prox1* gene. Abbreviations: *Efl=Ephydatia fluviatilis*; *Em=Ephydatia muelleri*; *Sl=Spongilla lacustris; Efr=Eunapius fragilis*; *Th=Trochospongilla horrida*; - = negative control without RNA template; M=molecular-size marker. Arrows indicate the size in bp of the amplified products for each gene.

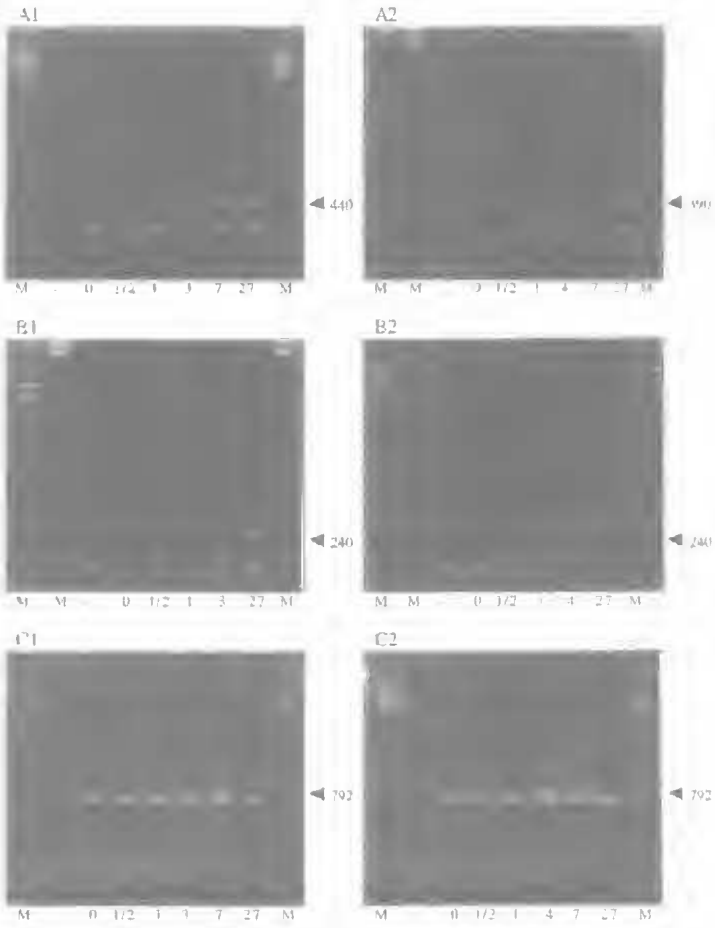

FIG.2. Expression of homeobox-containing genes in the course of development. Amplified products of RT-PCR of total RNA isolated from gemmules to the formation of fully functional sponges. A,Expression of *EmH-3*. B, Expression of *prox1*. C, Control, expression of *EmA 1* (Actin gene from *E. muelleri*). 1=*E. muelleri*; 2=*E. fluviatilis*. Developmental stages are expressed as days after incubation at 20°C in mineral medium; Abbreviations: - =negative control without RNA template; M=molecular-size marker. Arrows indicate the size in bp of the amplified products for each gene.

approximately 440bp long, the same size as *E. muelleri* transcripts and about 50bp longer than *E. fluviatilis* transcripts. *E. fluviatilis* transcripts were 390bp long, the expected *prox2* transcript size according to Seimiya et al. (1994).

We noticed also that *prox1* was expressed at a slightly lower level in *E. fragilis* (Fig. 1B).

The study of the temporal expression of *EmH-3*, *prox1*, and *EmA 1*, summarised in Figure 2, reveals a clear-cut difference in the level and pattern of expression of these genes, though the absolute amounts of expression cannot been directly compared from one gene to another because of possible differences in amplification efficiency between the different sets of primers.

EmH-3 gene was expressed differentially in the course of development in both species (Fig. 2, A1 and A2). In gemmules, transcripts were present in very small amounts as they were almost undetectable by RT-PCR. The level of expression increased very slightly until hatching, 3 days and 4 days of incubation, respectively. At that time, a high level of expression was observed. This level was maintained during several days, even in sponges transferred to the field for three weeks (27-day-old sponges).

On the other hand, *prox1* gene appeared to be expressed at nearly the same level throughout development: transcripts were already discernible in the gemmules and their level varied little although a slight enhancement could be detected at the moment of hatching (Fig. 2, B1 and B2).

In the control set of experiments, *EmA 1* Actin gene from *E. muelleri* (Ducy, 1993), was strongly expressed at all stages of development (Fig. 2, C1 and C2).

In HU-treated sponges, the evolution of the expression of *EmH-3* was roughly the same as in non-treated sponges (Fig. 3). The level of transcripts, very low during the first days of incubation reached already high values one day before hatching (6th day of incubation). Actin expression was high at all stages.

DISCUSSION

The results of the RT-PCR survey of *EmH-3* and *prox1* in 5 freshwater species corroborate previous Southern hybridisations realised with *EfH-1* as a probe (Richelle et al., 1995). They indicate that *S. lacustris*, *E. fragilis* and *T.*

FIG. 3. Expression of *EmH-3* in hydroxyurea-treated sponges in the course of development. Amplified products of RT-PCR of total RNA isolated from gemmules to the formation of fully functional sponges in *E. fluviatilis*. Developmental stages are expressed as days after incubation at 20°C in mineral medium. Abbreviations: A=Expression of *EmA 1* (Actin gene from *E. muelleri*); -=negative control without RNA template; M=molecular-size marker. Arrows indicate the size in bp of the amplified products for each gene.

horrida possess an *EmH-3-like* gene but that this gene differs in structure from the *E. fluviatilis EfH-1/ prox2* gene. This is clearly evidenced by the difference in length of their transcripts. This difference could be explained by a differential splicing as is the case for *E. muelleri* the first exon of which is 54bp longer than that of *E. fluviatilis* (Richelle et al., 1998).

The presence of an *EmH-3*-like gene in 5 species of freshwater sponges together with the high identity of sequence with *SpoxTA1* from *Tethya aurantia*, a marine sponge, may indicate that this type of gene could be widespread among Porifera and could represent one of their ancestral homeobox-containing gene. This hypothesis is supported by the data of Larroux & Degnan (1999), showing that a *prox2*-like gene is present in two other marine sponge species, *Iotrocota baculifera* and *Tedania digitata*.

On the contrary, prox1 although present in the 5 species of freshwater sponges, does not show a high degree of similarity with other sponge homeobox-containing genes isolated to date.

The temporal pattern of expression of *EmH-3* clearly demonstrates a differential expression of *EmH3* gene during gemmule germination and hatching. The enhancement of the expression at the moment of hatching suggests that this gene is particularly involved at that stage of development and provides evidence for a role in cell-fate decisions during differentiation. Actually, at hatching, all cells began to differentiate from the undifferentiated gemmular archaeocytes in a definite sequence: first the pinacocytes and the sclerocytes, then the choanocytes which arise by repeated divisions undergone by the archaeoeytes (Rasmont & Rozenfeld, 1981). The persistence of the expression of *EmH-3* in the adult sponge is probably related to the continuous replacement and/ or differentiation of cells occurring in the organism, in particular the turnover of the choanocytes (Rozenfeld & Rasmont, 1976).

In their work, Seimiya et al. (1994) concluded that *prox2* transcripts were identified at all stages of differentiation in *E. fluviatilis*. This discrepancy with our results arises from the fact that the authors studied only one undefined stage before hatching and that obviously, as demonstrated by our results, the main events occur during gemmule hatching.

On the other hand, the kinetics of expression of *prox1* show that this gene is expressed almost at the same level at all stages of development in *E. muelleri* as in *E. fluviatilis*.

In HU-treated sponges, the overall pattern of expression of *EmH-3* is similar to that in untreated sponges. The time-dependent increase in expression of *EmH-3* is not delayed in the presence of hydroxyurea, even though hatching is delayed to day 6 rather than day 4. Thus, in contrast to control sponges, the increased expression of *EmH-3* in HU-treated sponges appears to precede hatching, since it occurs before the migration of the cells through the micropyle. Consequently, it would be interesting to determine if differentiation processes observed in control sponges at hatching have already started in unhatched HU-treated gemmules.

These experiments are of special interest because hydroxyurea inhibit the differentiation of only one type of cells, i.e. the choanocytes, the other cell types being insensitive to its action. In addition, HU-blocked sponges provide a suitable source for the isolation of pure populations of embryonic archaeocytes that can be brought to differentiate and achieve normal development by removal of hydroxyurea from the medium (Rozenfeld & Rasmont, 1976).

Indeed, to gain more understanding of the role played by *EmH-3* and *prox1* in sponge development, it would be essential to determine what

happens when archaeocytes differentiate into other cells, in particular into choanocytes but also to determine in which cells these genes are expressed.

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